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*Cancer Therapy: Preclinical*

# Expression of t-DARPP Mediates Trastuzumab Resistance in Breast Cancer Cells

Abbes Belkhiri,[1] Altaf A. Dar,[1] DunFa Peng,[1] Mohammad H. Razvi,[1] Cammie Rinehart,[2] Carlos L. Arteaga,[2,3,4] and Wael El-Rifai[1,3,4]

**Abstract**

**Purpose:** We have investigated the role of t-DARPP in trastuzumab resistance in ERBB2-amplified and overexpressed breast cancer cell lines.

**Experimental Design:** We have used the HR-5 and HR-6 trastuzumab-resistant cells that were established from tumors that recurred in the presence of trastuzumab therapy following xenografts of BT-474 cells in nude mice. In addition, SKBR-3 cells, engineered for stable expression of t-DARPP, and HCC-1569 cells, which have constitutive expression of t-DARPP and are *de novo* resistant to trastuzumab, were used.

**Results:** We reported ≥15-fold up-regulation of mRNA and protein levels of t-DARPP in HR-5 and HR-6 cells compared with their progenitor BT-474 trastuzumab-sensitive cells. The t-DARPP expression was not regulated by changes in its promoter DNA methylation levels. The SKBR-3 cells stably expressing t-DARPP developed resistance to trastuzumab compared with their parental cells and empty vector controls ($P < 0.01$). The trastuzumab-resistant cell lines showed a significant increase in pAKT (Ser$^{473}$) and BCL2 protein levels. The small interfering RNA knockdown of t-DARPP in all trastuzumab-resistant cells led to a significant reduction in ERBB2, pAKT (Ser$^{473}$), and BCL2 protein levels with a significant decrease in cell viability ($P ≤ 0.001$) and an increase in cleaved caspase-3 levels, indicating the progression of these cells toward apoptosis. The t-DARPP protein was associated with both heat shock protein 90 and ERBB2 forming a potential protein complex. This association may play a role in regulating ERBB2 protein in trastuzumab-resistant cells.

**Conclusion:** We conclude that t-DARPP is a novel molecular target that can mediate the therapeutic resistance to trastuzumab in breast cancer cells.

Amplification of ERBB2 is the most common mechanism for ERBB2 activation in breast cancer (1, 2). This amplification occurs in ~25% of invasive breast cancers and is associated with poor patient outcome. The *ERBB2* oncogene is a member of the epidermal growth factor receptor family and encodes a transmembrane tyrosine kinase receptor. The amplification and expression of ERBB2 have been linked to prognosis and response to therapy with the anti-HER-2-humanized monoclonal antibody, trastuzumab (Herceptin; Genentech), in patients with advanced metastatic breast cancer (3). However, one of the major clinical problems encountered with trastuzumab treatment is that metastatic breast cancer patients, who initially responded to trastuzumab, showed disease progression within 1 year from treatment initiation (4). Preclinical studies have indicated that increased signaling via the phosphatidylinositol 3-kinase/AKT pathway may contribute to trastuzumab resistance (5, 6).

*PPP1R1B*, also known as dopamine and cyclic AMP–regulated phosphoprotein (*DARPP-32*), is involved in dopaminergic neurotransmission and is a major factor in the functioning of dopaminoceptive neurons (7). Through cloning and physical mapping strategies of transcripts in the *ERBB2* amplicon region, we have identified a transcriptional splice variant of *DARPP-32* that encodes a truncated protein isoform, which we named *t-DARPP* (accession no. AY070271; ref. 8). t-DARPP lacks the NH$_2$-terminal protein phosphatase inhibitory domain of DARPP-32 and is frequently overexpressed in several adenocarcinomas (8). In this report, we have investigated the role of t-DARPP in trastuzumab resistance in breast cancer.

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## Materials and Methods

*Cell lines and trastuzumab.* The human breast cancer cell lines, BT-474, SKBR-3, and HCC-1569 cells, were purchased from the American Type Tissue Culture Collection. The BT-474 and SKBR-3 cells are sensitive, whereas the HCC-1569 is *de novo* resistant to trastuzumab (9). To obtain trastuzumab-resistant cell line model (HR), the BT-474 cells
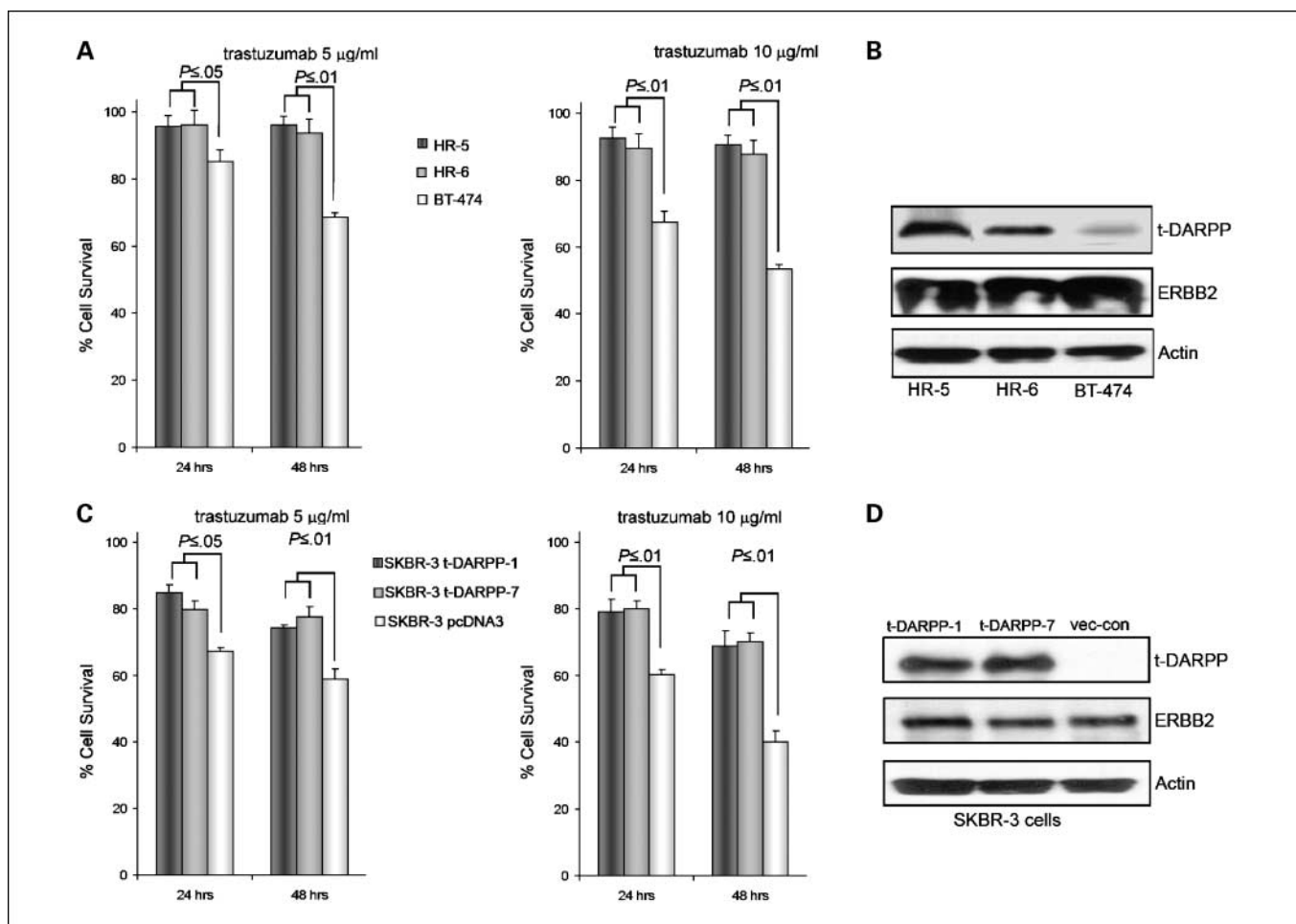
**Fig. 1.** t-DARPP overexpression correlates with trastuzumab resistance and increased cell survival. Cells were treated with vehicle or trastuzumab (5 and 10 μg/mL) for 24 and 48 h and then subjected to Cell Titer-Glo Luminescent Cell Viability Assay (Promega). *A*, cell survival was significantly lower in the parental BT-474 cell line compared with trastuzumab-resistant HR-5 and HR-6 cells for all tested trastuzumab concentrations and time points. *B*, protein extracts from nontreated cells (BT-474, HR-5, and HR-6) were subjected to Western blot analysis of ERBB2 and t-DARPP. ERBB2 protein levels were comparable in all cells. However, t-DARPP protein levels were dramatically higher in HR-5 and HR-6 compared with BT-474 cells. *C*, similar to trastuzumab-resistant HR-5 and HR-6 cells (*A*), cell survival of SKBR-3 cells stably expressing t-DARPP (clones 1 and 7) was also markedly higher than control SKBR-3 cells following treatment with trastuzumab. *D*, protein extracts from nontreated SKBR-3-t-DARPP-1, SKBR-3-t-DARPP-7, and control SKBR-3-pcDNA3 cells were subjected to immunoblotting of ERBB2 and t-DARPP. As expected, t-DARPP protein levels were significantly higher in SKBR-3-t-DARPP-1 and SKBR-3-t-DARPP-7 clones compared with SKBR-3-pcDNA3 control cell line. ERBB2 protein levels were comparable in all tested cells. Gel loading was normalized for equal β-actin.

were established as xenografts in athymic nude mice and HR cell lines were generated from tumors that recurred in the presence of antibody therapy (10). The isolated cells (HR-5 and HR-6) maintained resistance to trastuzumab in culture and *in vivo* when reinjected into nude mice (for details, see ref. 10). All cells were maintained in improved MEM (Life Technologies) containing 10% FCS (Hyclone) at 37°C in a humidified 5% $CO_2$ atmosphere.

*Vectors.* The expression plasmid for t-DARPP was generated by PCR amplification of the full-length coding sequence of t-DARPP (accession no. AY070271) and cloned in-frame into pcDNA3.1 (Invitrogen Life Technologies). Stably transfected SKBR-3 cells expressing t-DARPP or pcDNA3.1 empty vector were generated following standard protocols as described previously (11). After selection with 400 μg/mL neomycin (Invitrogen Life Technologies), clones were screened for t-DARPP protein expression by Western blot analysis.

*Cell viability and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays.* Cells (5 × $10^3$ per well) were seeded onto a 96-well plate. The survival of these cells after treatment with vehicle, trastuzumab, or knockdown of t-DARPP was determined using the Cell Titer-Glo Luminescent Cell Viability Assay Kit (Promega)

following the supplier's instructions. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was done as described previously (11) using *In Situ* Cell Death Detection Kit, TMR red (Roche Diagnostics).

*Immunoblot analysis.* Cell lysates (10 μg/lane) were separated by 10% SDS-PAGE and subjected to immunoblot analysis. Gel loading was normalized for equal β-actin. Proteins were then transferred onto Hybond-P polyvinylidene difluoride membranes (Amersham Biosciences). Horseradish peroxidase–conjugated secondary antibodies were obtained from Amersham Biosciences. Immunoreactive bands were visualized by enhanced chemiluminescence (Pierce). A COOH-terminal antibody that recognizes t-DARPP was obtained from Santa Cruz Biotechnology. ERBB2, AKT, pAKT ($Ser^{473}$), cleaved caspase-3, heat shock protein 90 (HSP90), and β-actin antibodies were obtained from Cell Signaling.

*Immunoprecipitation.* The trastuzumab-resistant HR-5 cells were washed twice with ice-cold PBS and solubilized for 30 min at 4°C with lysis buffer (1% Triton X-100) containing 1% Halt protease inhibitor cocktail (Pierce Biotechnology). The cell lysates were first sonicated and then spun down at 15,000 rpm for 10 min. The

supernatants were collected and protein concentration was measured by standard Bradford assay. Total protein (200 µg) was incubated with 1 µg anti-ERBB2, anti-t-DARPP, or anti-HSP90 antibodies overnight at 4°C on a rotating platform followed by incubation with 50 µL protein G-agarose beads (Roche Diagnostics) for 24 h at 4°C on a rotating platform. The beads were spun down at 15,000 rpm for 5 min and washed three times with ice-cold PBS. The immunoprecipitated protein was eluted by resuspending the beads in 60 µL of 2× sample buffer and incubating at 95°C for 5 min. The eluted protein was resolved using 10% SDS-PAGE and transferred onto Hybond-P polyvinylidene difluoride membranes for Western blot analysis of ERBB2, t-DARPP, and HSP90 proteins.

*Quantitative real-time PCR.* Quantitative real-time PCR was done using an iCycler (Bio-Rad) with a threshold cycle number determined by use of iCycler software version 3.0. Single-stranded cDNA was synthesized using the Advantage Reverse Transcription-PCR Kit (Clontech). Gene-specific primers for DNA and mRNA-specific sequences of *t-DARPP, ERBB2, β-actin*, and *HPRT1* were designed, and the results were normalized to *β-actin* and *HPRT1* as stable reference genes for quantitative real-time PCR. All primer sequences are available upon request. The results were normalized and fold amplification and expression were calculated as described previously (8).

*Gene expression knockdown by small interfering RNA.* The BT-474, HR-5, HR-6, SKBR-3-t-DARPP-1, SKBR-3-t-DARPP-7, and HCC-1569 cells were transfected with control small interfering RNA (siRNA; sc-37007) or t-DARPP siRNA (sc-35173) using siRNA transfection reagent (sc-29528) and transfection medium (sc-36868) following the manufacturer's instructions (Santa Cruz Biotechnology).
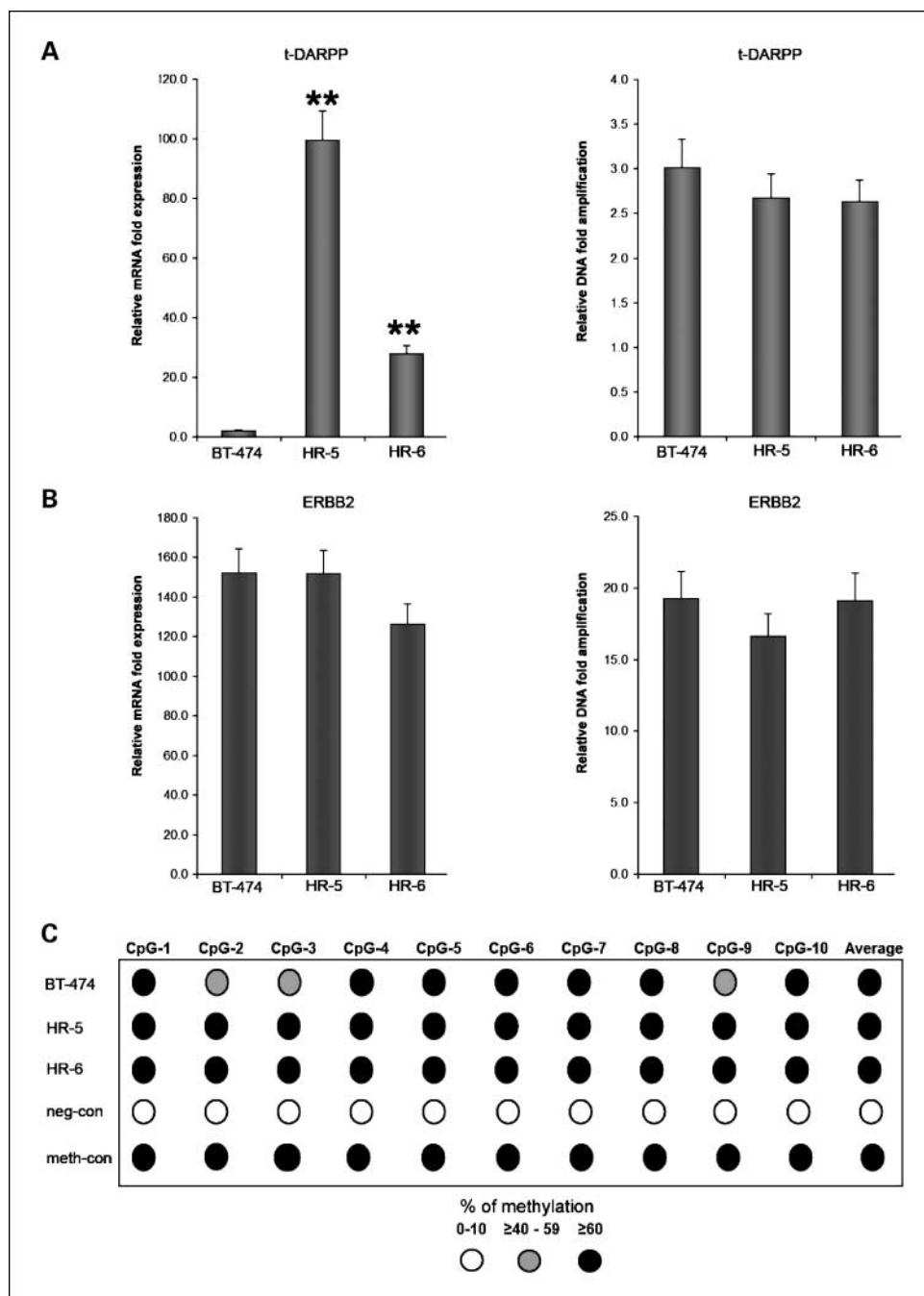


**Fig. 2.** Transcriptional up-regulation of t-DARPP in trastuzumab-resistant cell lines is not associated with gene amplification or promoter hypomethylation. Gene-specific primers for DNA- and mRNA-specific sequences of *t-DARPP, ERBB2, β-actin*, and *HPRT1* were used for PCR and real-time PCR in BT-474, HR-5, and HR-6 cells; the results were normalized to *β-actin* and *HPRT1*. *A,* t-DARPP mRNA expression levels in HR-5 and HR-6 cells were 25- to 100-fold higher compared with BT-474 cells (*, $P \leq 0.001$). In contrast, t-DARPP gene amplification levels were similar in all cells. *B,* ERBB2 mRNA expression and gene amplification levels remained unchanged in all cells. *C,* DNA bisulfite treatment and pyrosequencing analysis of DNA methylation. DNA from BT-474, HR-5, and HR-6 cells was extracted and modified by bisulfite treatment as described in Materials and Methods. A CpG island from -1,438 to -830 of t-DARPP was amplified with PCR using specific primers. The PCR products were then subjected to pyrosequencing analysis to determine DNA methylation of 10 CpG sites within -1,161 to -1,109 region of t-DARPP. Comparable levels of DNA methylation were detected in all three cell lines, indicating that DNA methylation of t-DARPP CpG island does not regulate its expression in these cell lines.
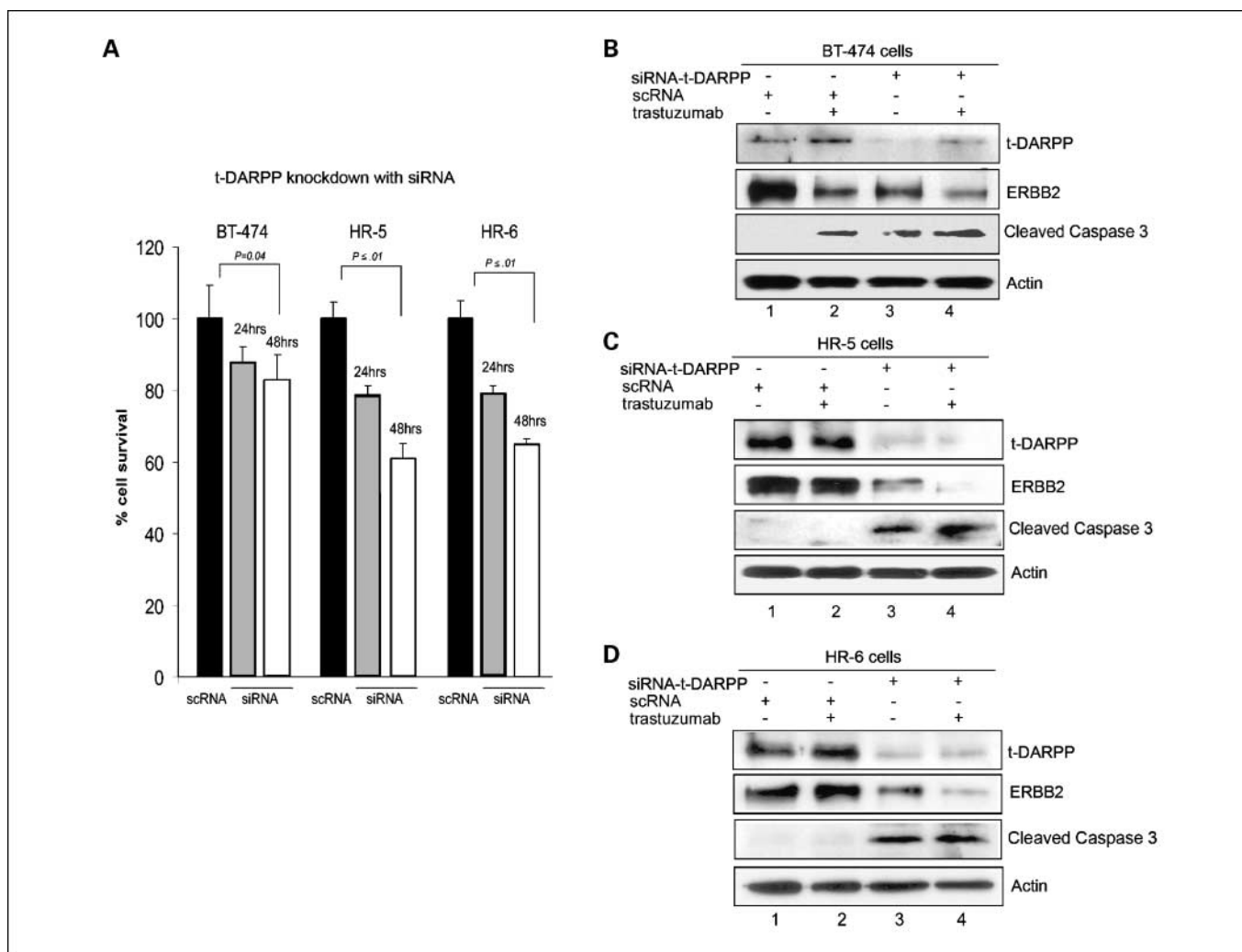
**Fig. 3.** Knockdown of t-DARPP induces apoptosis in trastuzumab-resistant breast cancer cells. *A*, BT-474, HR-5, and HR-6 cells were transfected with control scrambled siRNA or t-DARPP siRNA oligonucleotides and then subjected to Cell Titer-Glo Luminescent Cell Viability Assay 24 and 48 h after transfection. Overall, cell survival was significantly lower in all cells transfected with t-DARPP siRNA compared with control cells. In addition, cell survival of HR-5 and HR-6 is dramatically lower after knockdown of t-DARPP ($P \leq 0.01$). *B* to *D,* cells were transfected with control scrambled siRNA or t-DARPP siRNA oligonucleotides. The cells were then treated 24 h after transfection with 20 μg/mL trastuzumab or vehicle (control) for 48 h. *B,* protein extracts from BT-474 cells (trastuzumab-sensitive) were subjected to Western blot analysis of t-DARPP, ERBB2, and caspase-3. As expected, trastuzumab alone down-regulated ERBB2 protein level and induced apoptosis as indicated by appearance of cleaved caspase-3 fragment (*lane 2*). Knockdown of t-DARPP alone (*lane 3*) and in combination with trastuzumab (*lane 4*) significantly down-regulated ERBB2 and activated caspase-3. *C,* protein extracts from HR-5 cells (trastuzumab-resistant) were subjected to immunoblotting of t-DARPP, ERBB2, and caspase-3. As expected, trastuzumab alone neither down-regulated ERBB2 protein level nor activated caspase-3 (*lane 2*). Knockdown of t-DARPP alone (*lane 3*) and in combination with trastuzumab (*lane 4*) down-regulated ERBB2 and activated caspase-3. *D,* protein extracts from HR-6 cells (trastuzumab-resistant) were subjected to Western blot analysis of t-DARPP, ERBB2, and caspase-3. As in HR-5 cells (*B*), trastuzumab alone did not down-regulate ERBB2 or activate caspase-3 (*lane 2*). In contrast, knockdown of t-DARPP alone (*lane 3*) or in combination with trastuzumab (*lane 4*) markedly down-regulated ERBB2 and activated caspase-3. Gel loading was normalized for equal β-actin.

*DNA bisulfite treatment and pyrosequencing analysis of DNA methylation.* A CpG island was found in t-DARPP promoter from -1,438 to -830 of transcription start site. A pyrosequencing assay was designed using PSQ assay design software (Biotage), which quantitatively detects DNA methylation level of 10 CpG sites within -1,161 to -1,109 of t-DARPP promoter. The PCR primers sequences were 5′-GTTTGAGAGGGGGGAGTTTAGTAT-3′ and 5′/bio/-TAAAACAC-CAACCCCTTACCAT-3′. A sequencing primer (5′-GAGAGGGGGAGTT-TAGTATA-3′) was used to read the 10 CpG sites. All primers were purchased from Integrated DNA Technologies. DNA from three cell lines (BT-474, HR-5, and HR-6) was purified using DNeasy kit (Qiagen) and 1 μg was modified by bisulfite using EZ DNA Methylation-Gold Kit (ZYMO Research) according to the manufacturer's protocol. Following PCR amplification, the products were subjected to quantitative pyrosequencing analyses using Biotage PyroMark MD System (Biotage) according the protocol provided by the manufacturer.

## Results

*Expression of t-DARPP and trastuzumab resistance.* Trastuzumab-sensitive BT-474 and SKBR-3 human breast cancer cells are known to display DNA amplification and mRNA and protein overexpression of ERBB2 (9, 12). Our findings confirmed earlier report (10) and showed the resistance to trastuzumab in HR-5 and HR-6 cells as shown by increased cell survival following treatment with trastuzumab (5 and 10 μg/mL; Fig. 1A). Interestingly, t-DARPP protein levels were considerably higher in HR-5 and HR-6 cells compared with their parental BT-474 cells (Fig. 1B), whereas the ERBB2 protein levels remained unchanged in all cells (Fig. 1B). These results suggest a selective overexpression of t-DARPP with increased cell survival and resistance to trastuzumab in HR

cells. In addition, the SKBR-3-t-DARPP stably expressing cells (clones 1 and 7) developed significant resistance to trastuzumab compared with the empty vector control SKBR-3-pcDNA3 cells (Fig. 1C). The immunoblotting indicated that ERBB2 protein levels were comparable in all cells (Fig. 1D). To determine whether t-DARPP was transcriptionally up-regulated and associated with gene amplification, we did quantitative real-time PCR of t-DARPP. The results showed 25- to 100-fold up-regulation of the t-DARPP transcript levels in HR-5 and HR-6 clones compared with the BT-474 parental cell line (Fig. 2A). In contrast, t-DARPP relative DNA amplification was similar in all cell lines, showing only 3-fold DNA amplification compared with normal controls (Fig. 2A). On the other hand, ERBB2 mRNA overexpression and DNA amplification levels were comparable in BT-474, HR-5, and HR-6 cells (Fig. 2B). These results indicate that DNA amplification of t-DARPP was not driving its mRNA overexpression and suggest that other mechanisms are involved in its transcription up-regulation in HR cells. We investigated if a methylation-dependent epigenetic mechanism could be involved in the transcriptional regulation of t-DARPP in BT-474 and HR cells where it may become hypomethylated in HR cells compared with BT-474 cells. The quantitative analysis of DNA methylation levels in 10 CpG sites in the t-DARPP promoter ruled out this possibility and indicated comparable levels of DNA methylation in all cells (Fig. 2C). Taken together, our results indicate that changes in DNA copy numbers and/or promoter methylation of t-DARPP are not the underlying molecular mechanisms for its transcription up-regulation in HR cell model. Further studies are required to identify the transcription regulators of t-DARPP.

*t-DARPP mediates cell survival and its knockdown induces cell death in trastuzumab-resistant cells.* We did a knockdown of t-DARPP in BT-474 and HR cells to ascertain its role in trastuzumab resistance. Indeed, knockdown of t-DARPP in HR cells led to a significant reduction in cell survival compared with scrambled siRNA control ($P \leq 0.001$; Fig. 3A). The BT-474 cells showed a slight reduction in cell survival, following its knockdown, and this correlated with their low expression level of t-DARPP (Fig. 1B). Furthermore, the knockdown of t-DARPP, alone or in combination with trastuzumab treatment, led to a reduction in ERBB2 levels and induced apoptosis as

indicated by the activated caspase-3 signal (Fig. 3B-D). In contrast, trastuzumab alone did not down-regulate ERBB2 or activate caspase-3 in HR cells (Fig. 3C and D). To further confirm induction of apoptosis following knockdown of t-DARPP in HR cells, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was employed on HR-6 cells. The results confirmed resistance of HR-6 cells to trastuzumab. The knockdown of t-DARPP in HR-6 cells led to >8-fold increase in apoptosis ($P < 0.001$; Fig. 4). The combination of t-DARPP knockdown together with trastuzumab treatment showed a similar effect as using t-DARPP knockdown alone (Fig. 4). Taken together, these results indicate that knockdown of t-DARPP alone was sufficient to down-regulate ERBB2 and induce apoptosis in HR-5 and HR-6 trastuzumab-resistant cells.

*Overexpression of t-DARPP in trastuzumab-resistant cells leads to increased phosphorylation of AKT and BCL2 protein levels.* Our results show a significant increase of pAKT (Ser[473]) and BCL2 protein levels in HR-5 and HR-6 cells (Fig. 5A and B) and SKBR-3-t-DARPP-1 and SKBR-3-t-DARPP-7 clones (Fig. 5C and D) compared with their control cells. The knockdown of t-DARPP in these cells led to a dramatic down-regulation of ERBB2 protein associated with a notable decrease of pAKT (Ser[473]) without affecting total AKT protein level (Fig. 5B and D). In addition, the prosurvival protein BCL2 was also down-regulated in the two trastuzumab-resistant cell models following the knockdown of t-DARPP (Fig. 5B and D). As HSP90 was reported to stabilize expression of ERBB2 through binding (13), we investigated whether t-DARPP could regulate the expression of HSP90. Our results indicate that HSP90 levels remained similar in all cells and did not change following t-DARPP knockdown (Fig. 5A-D). We have further investigated the expression of t-DARPP in HCC-1569 that amplify and overexpress ERBB2 and have *de novo* resistance to trastuzumab (9). The results indicated that HCC-1569 has a significant constitutive overexpression of t-DARPP compared with the trastuzumab-sensitive parental BT-473 and SKBR-3 cells (Fig. 5). The HCC-1569 cell line was developed from a patient with a metastatic tumor who received prior chemotherapy (14). We used the HCC-1569 cells for further validation of our results. Indeed, the knockdown of t-DARPP in HCC-1569 cells led to a significant decrease of ERBB2, pAKT (Ser[473]), and BCL2 protein levels in addition to the activation of
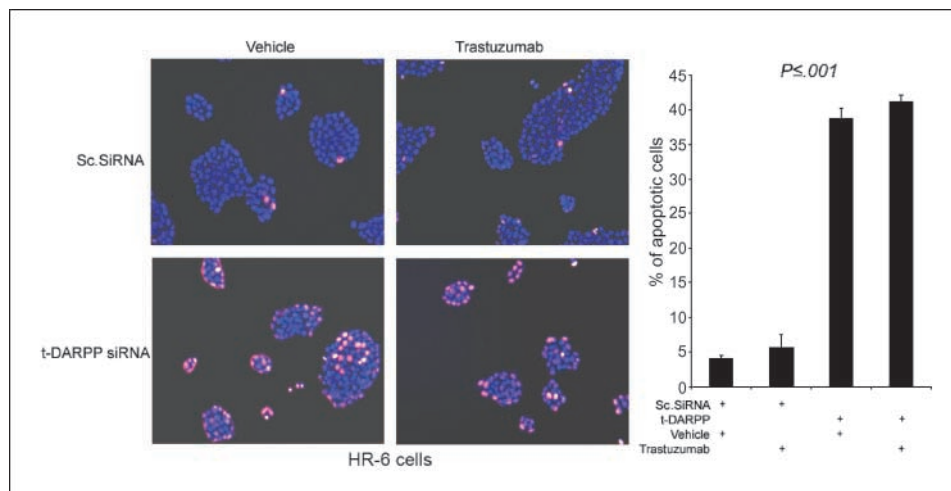


**Fig. 4.** Knockdown of t-DARPP induces apoptosis in trastuzumab-resistant cells. Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay was done on the trastuzumab-resistant HR-6 cells with scrambled siRNA or t-DARPP siRNA oligonucleotides following the treatment with 20 μg/mL trastuzumab or vehicle (PBS) for 24 h. The cells were counterstained with 4′,6-diamidino-2-phenylindole (blue fluorescence) and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling–positive apoptotic cells were visualized with TRITC filter (red fluorescence). Two hundred cells from 20 random fields at ×40 (>400 cells) were counted. The percentage of apoptosis was determined relative to the total 4′,6-diamidino-2-phenylindole–positive cells. As shown, the results show resistance of HR-6 cells to trastuzumab and >8-fold increase in apoptosis following the knockdown of t-DARPP ($P \leq 0.001$).

**Fig. 5.** t-DARPP activates the phosphatidylinositol 3-kinase/AKT survival pathway in trastuzumab-resistant cells. *A,* protein extracts from BT-474, HR-5, and HR-6 cells were analyzed by Western blot for t-DARPP, pAKT (Ser[473]), AKT, BCL2, and HSP90. *B,* proteins from HR-5 and HR-6 cells transfected with control scrambled siRNA or t-DARPP siRNA oligonucleotides were also subjected to Western blot analysis of ERBB2 in addition to the proteins described above. pAKT and BCL2 protein levels were markedly higher in HR-5 and HR-6 compared with BT-474 control cells. HSP90 protein levels remained unchanged. Knockdown of t-DARPP in HR-5 and HR-6 cells dramatically decreased ERBB2 and BCL2 protein levels and pAKT but did not affect HSP90 levels. *C,* protein extracts from SKBR-3-pCDNA3 (*vec-con*), SKBR-3-t-DARPP-1, and SKBR-3-t-DARPP-7 cells were subjected to Western blot analysis of t-DARPP, AKT, pAKT (Ser[473]), BCL2, and HSP90. *D,* protein extracts from SKBR-3-t-DARPP-1 and SKBR-3-t-DARPP-7 cells transfected with control scrambled siRNA or t-DARPP siRNA oligonucleotides were also analyzed by Western blot for ERBB2 and the other proteins described above. As in *A,* pAKT and BCL2 protein levels were significantly higher in SKBR-3-t-DARPP-1 and SKBR-3-t-DARPP-7 than control cells. Moreover, HSP90 protein levels remained equal in all cells. Knockdown of t-DARPP in SKBR-3-t-DARPP-1 and SKBR-3-t-DARPP-7 cells markedly decreased pAKT, BCL2, and ERBB2 protein levels compared with control cells. Gel loading was normalized for equal β-actin. *E,* protein extracts from HCC-1569 cells, *de novo* resistant to trastuzumab, transfected with scrambled siRNA or t-DARPP siRNA oligonucleotides were also analyzed by Western blot for t-DARPP, ERBB2, HSP90, AKT, pAKT (Ser[473]), BCL2, and caspase-3. As shown in *B* and *D*, knockdown of t-DARPP significantly decreased the ERBB2, pAKT (Ser[473]), and BCL2 protein levels. In addition, HSP90 remained unchanged and caspase-3 was activated.
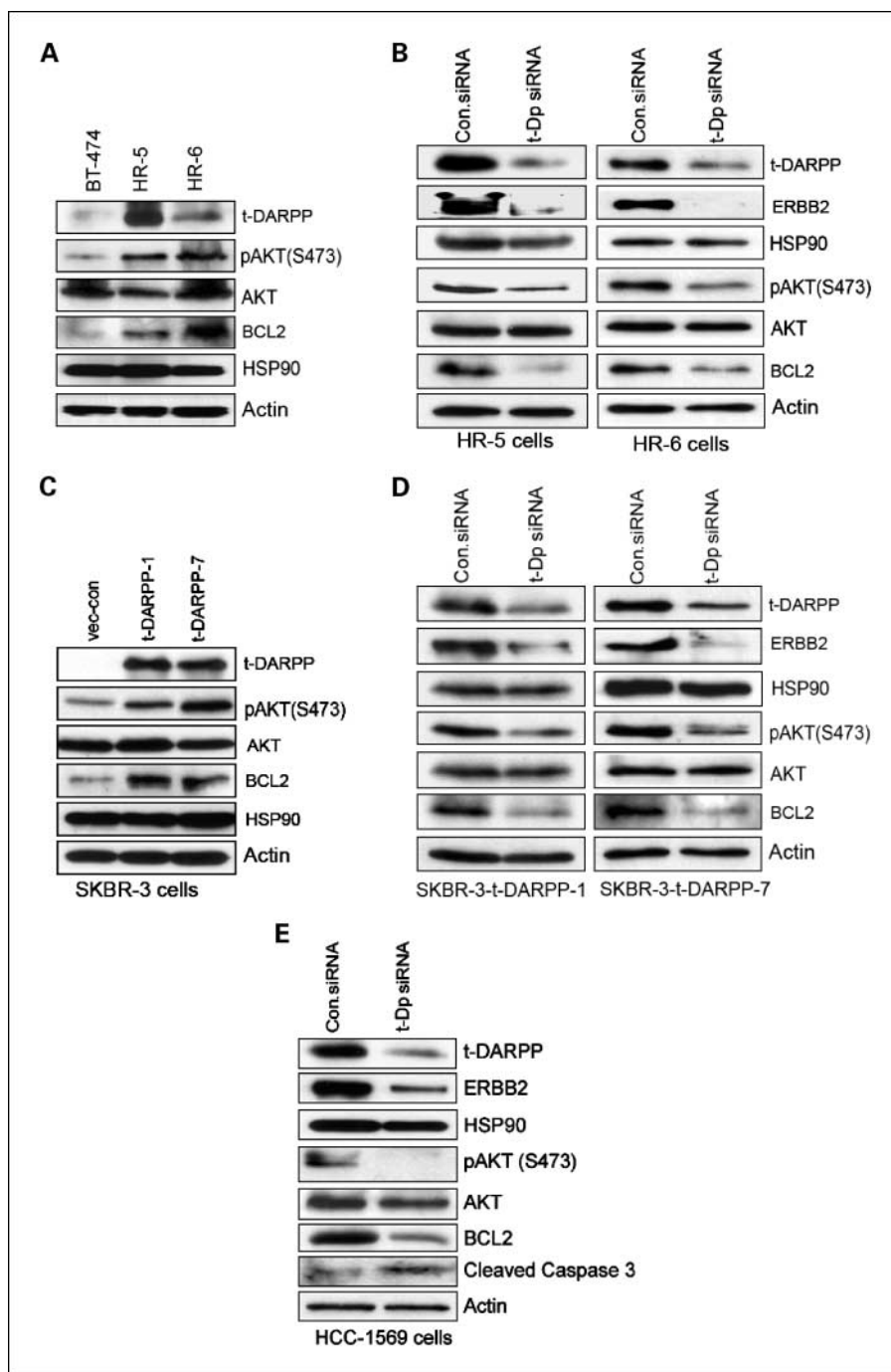
caspase-3 (Fig. 5E), confirming our results shown for HR and SKBR-3 cell models (Fig. 5A and D).

**t-DARPP is associated with HSP90 and ERBB2 proteins in trastuzumab-resistant cells.** To test for a possible protein-protein association between t-DARPP and ERBB2, we did coimmunoprecipitation experiments in which we immunoprecipitated endogenous ERBB2 from the trastuzumab-resistant cells (HR-5) and checked for the presence of t-DARPP. The reverse experiment was also done by immunoprecipitating t-DARPP from the same cells and detection of ERBB2 in the precipitates. We found that t-DARPP was indeed a component of the protein complex immunoprecipitated by ERBB2 anti-

body (Fig. 6). Immunoprecipitation by t-DARPP antibody also indicated that ERBB2 formed a protein complex with t-DARPP (Fig. 6). As HSP90 was reported to bind and stabilize ERBB2 protein (13), we further investigated if t-DARPP could associate with HSP90 and form a complex with ERBB2. The results showed that t-DARPP associated with HSP90 (Fig. 6). We confirmed ERBB2 association with HSP90 in HR-5 cells as indicated by coimmunoprecipitation with either HSP90 or ERBB2 antibodies (Fig. 6). Taken together, these results indicate that t-DARPP, ERBB2, and HSP90 associate with one another, forming a protein complex in trastuzumab-resistant breast cancer cells.

## Discussion

In this study, we have identified t-DARPP as a novel molecular target that leads to trastuzumab resistance in human breast cancer cells. The full-length DARPP-32 is a neuronally characterized protein that is centrally involved in dopamine-induced signaling pathways in the brain and is best known as a potent inhibitor of phosphatase I in neurosignaling. On the other hand, t-DARPP was recently cloned as a cancer-specific transcriptional splice variant of DARPP-32 that encodes a truncated protein of DARPP-32 (11). Whereas DARPP-32 encodes 204 amino acids, t-DARPP encodes 168 amino acids and lacks the $NH_2$-terminal protein phosphatase domain that is present in DARPP-32 (11). The molecular signaling and protein interactions of t-DARPP remain largely unexplored.

The BT-474 and SKBR-3 breast cancer cells are known to display DNA amplification and mRNA and protein over-expression of ERBB2 (12). These cells are ERBB2 dependent, where the inhibition of ERBB2 leads to growth arrest and/or tumor cell death both *in vitro* and *in vivo* (15). We have recently developed the HR-5 and HR-6 trastuzumab-resistant cells (C.L.A.) from their progenitor BT-474 through continuous exposure to trastuzumab and passaging *in vivo* (10). In this study, we show a significant overexpression of t-DARPP in HR-5 and HR-6 cells compared with BT-474 cells. DNA amplification and/or promoter DNA hypomethylation are known to mediate overexpression of several oncogenes (16, 17). Interestingly, the DNA copy numbers and promoter DNA methylation of t-DARPP remained unaffected in our model. These findings suggest that t-DARPP regulation in HR cells was not dependent on their DNA amplification or promoter methylation levels. The transcriptional regulatory mechanism(s) of t-DARPP remains unknown in the literature and future studies in this direction can unveil these mechanisms.

Several mechanisms of trastuzumab action have been reported [reviewed by Nahta et al. (4)]. Our results showed that the HR-5 and HR-6 cells constitutively expressing t-DARPP and the SKBR-3 cells stably expressing t-DARPP showed increased cell survival following treatment with trastuzumab compared with their controls. This finding underscored the possibility that t-DARPP expression is a novel molecular event that contributes to trastuzumab resistance. The concept of oncogene addiction was recently introduced as a mechanism where some cancers remain dependent on (addicted to) one or a few genes for both maintenance of the malignant phenotype and cell survival

(18). Our results from the *in vitro* model suggest that overexpression of t-DARPP was acquired following trastuzumab treatment and this could be due to the presence of a subpopulation of cancer cells that were selected for and replaced cell populations that were sensitive to trastuzumab. Therefore, it is possible that the HR cells became different from their parental BT-474 cells when they overexpressed t-DARRP and became dependent on its levels for their own survival.

Trastuzumab suppresses ERBB2-mediated activation of the phosphatidylinositol 3-kinase and mitogen-activated protein kinase signaling pathways, and this may be through internalization and degradation of the ERBB2 receptor (19) or as a result of disrupting the interaction between ERBB2 and the Src tyrosine kinase, which leads to activation of the phosphatidylinositol 3-kinase inhibitor PTEN (5). The failure of trastuzumab to inhibit the ERBB2-mediated activation of the phosphatidylinositol 3-kinase/AKT pathway has been suggested to play a role in trastuzumab resistance (5, 6). Our findings show that overexpression of t-DARPP in the two cell models (BT-474 and SKBR-3) led to increased phosphorylation of AKT and increased BCL2 protein levels. The knockdown of endogenous t-DARPP in the BT-474 cell model led to a significant reduction in cell survival. Interestingly, this effect was associated with a remarkable abrogation of ERBB2 protein levels and an induction of cleaved caspase-3, indicating progression of cells toward apoptosis. In addition, knockdown of t-DARPP resulted in a significant decrease of pAKT and BCL2 protein levels that can also explain the role of t-DARPP in cell survival in this particular cell model. We have recently shown that t-DARPP expression up-regulates BCL2 through CREB/ATF-dependent mechanism that requires active AKT in gastric cancer cells (20). BCL2 protein is a pivotal regulator of apoptotic cell death that counteracts drug-induced apoptosis and shifts the balance toward cancer cell survival through stabilization of the mitochondrial transmembrane potential and inhibition of cytochrome *c* release and activation of caspases (21). Consistent with our results, the knockdown of t-DARPP in an additional trastuzumab resistant cell line (HCC-1569) that has constitutive expression of t-DARPP confirmed the role of t-DARPP in regulating AKT and BCL2 in trastuzumab-resistant breast cancer cells. Our findings together with a recent report underscore the activity of the AKT pathway as a major determinant of trastuzumab resistance in breast cancer (6). The regulation of AKT pathway by t-DARPP could therefore be one of the mechanisms that are important in trastuzumab resistance. Further studies on
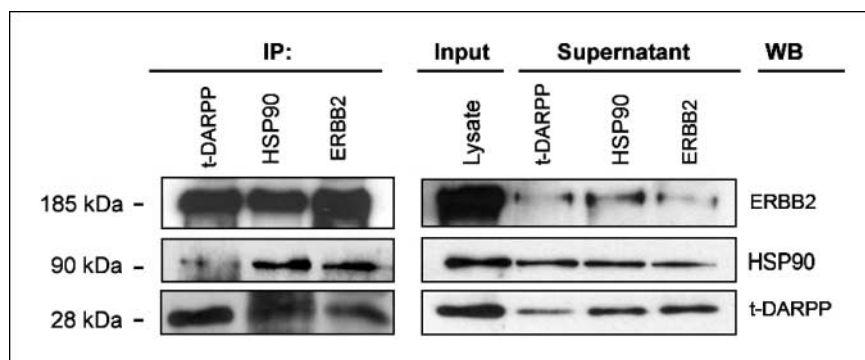


**Fig. 6.** t-DARPP associates with ERBB2 and HSP90 proteins in trastuzumab-resistant cells. Immunoprecipitated proteins with t-DARPP, HSP90, or ERBB2 antibodies and the cell lysate (*Input*) from HR-5 cells were resolved on 10% SDS-PAGE and transferred onto Hybond-P polyvinylidene difluoride membranes for Western blot analysis of t-DARPP, HSP90, and ERBB2 proteins. Protein supernatants collected after the first spin of agarose beads were used as controls. Both HSP90 and ERBB2 coprecipitated with t-DARPP.

primary tumors from patients under follow-up are necessary to determine the frequency of t-DARPP expression in trastuzumab-resistant tumors.

Our results also indicate that t-DARPP may contribute to trastuzumab resistance by blocking the trastuzumab effect on ERBB2 and maintaining its high levels in these cells. HSP90 is known to stabilize expression of several signaling kinases including AKT (22) and ERBB2 (13). Inhibition of HSP90 with geldanamycin was shown to down-regulate ERBB2 through its ubiquitination by the cochaperone CHIP (23, 24). In line with these facts, we have shown that t-DARPP associated with both ERBB2 and HSP90 forming a potential protein complex. This may suggest a critical role for t-DARPP in regulating ERBB2 stability in trastuzumab-resistant cells. Further studies are required to elucidate the potential molecular mechanisms. Interestingly, we have shown recently a significant statistical correlation between the mRNA levels in genes bounded by *DARPP-32* and *GRB7* and suggested that a functional oncogenomic region bounded by *DARPP-32* and *GRB7* is uniquely amplified and overexpressed in cancer (25). Indeed, a recent study has suggested an evolutionary recombination hotspot around GSDML-GSDM locus that is closely linked to the oncogenomic recombination hotspot around the *PPP1R1B-ERBB2-GRB7* amplicon (26). These results taken together may indicate that this linkage may have been conserved during evolution to maintain functional interactions as a need for cell survival.

In conclusion, our findings underscore an important role of t-DARPP in trastuzumab resistance in breast cancer lines. This finding needs to be confirmed in prospective clinical trial studies. Further investigations are under way to characterize the mechanisms that may be involved in regulating the ERBB2 protein levels by t-DARPP.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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